Coordinated regulation of transcription factors through Notch2 is an important mediator of mast cell fate

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Coordinated regulation of transcription factors through *Notch2* is an important mediator of mast cell fate

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Abstract

Mast cells are thought to participate in a wide variety of pathophysiological conditions. Mechanisms of regulation, however, of mast cell production and maturation are still to be elucidated. Mast cell developmental process is likely to be profoundly affected by cell-autonomous transcriptional regulators such as GATA family and CCAAT/enhancer binding protein (C/EBP) family members. Extracellular regulators such as stem cell factor and interleukin-3 have essential roles in basal and inducible mast cell generation, respectively. The relationship, however, between the extracellular signaling and cellular transcriptional control is unclear, and the trigger of the mast cell development remains elusive. Notch signaling plays a fundamental role in the lymphopoietic compartment, but its role in myeloid differentiation is less clear. Here we demonstrate that Notch signaling connects environmental cues and transcriptional control for mast cell fate decision. Delta1, an established Notch ligand, instructs bone marrow common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) toward mast cell lineage at the expense of other granulocyte-macrophage lineages, depending on the function of the *Notch2* gene. Notch2 signaling results in the upregulation of Hes-1 and GATA3, while simultaneous overexpression of these transcription factors remarkably biases the progenitor fate toward the mast

cell-containing colony-forming cells. C/EBPα mRNA was downregulated in myeloid progenitors as a consequence of Hes-1 overexpression, in agreement with the recent proposal that the downregulation of C/EBPα is necessary for mast cell fate determination.

Taken together, signaling through Notch2 determines the fate of myeloid progenitors toward mast cell-producing progenitors, via coordinately upregulating Hes-1 and GATA3.

Introduction

Mast cells are thought to participate in a wide variety of physiologic and pathologic processes In addition to their involvement in allergic disorders and protective immune responses to parasites, mast cells are important in a broader sense in both innate and acquired immunity (1, 2). Mast cells migrate from the bone marrow at an immature differentiation stage and complete the maturation process in the peripheral tissues under the influence of local growth and differentiation factors (2). These environmental cues tailor the mast cell phenotype to carry out functions specific to each peripheral tissue. The main factors influencing mast cell number and phenotype include stem cell factor (SCF), which is a ligand for c-Kit, interleukin-3 (IL-3), and T helper type II (Th2) -associated cytokines, such as IL-4 and IL-9 (2). Signal transduction through the phosphatidylinositol 3 kinase pathway, Ras-mitogen activated protein kinase pathway, Janus kinase-signal transducer and activators of transcription pathway (3), etc., in the presence of IL-3 and SCF, might coordinate the lineage-specific transcription factors, but there is a disconnect between our understanding of cytokine signaling and transcription factor regulation. The generation of mast cells has been shown to be

dependent on cooperative interplay between regulatory proteins: PU.1 upregulation, GATA2 upregulation (4), and C/EBPα downregulation (5).

Notch receptor-mediated signaling has a fundamental role in cell fate determination in a variety of animals. In the mammalian immune system, Notch signaling is involved in the commitment and differentiation of T cells, development of splenic marginal zone B cells, and differentiation and functional modulation of mature T cells (6, 7). Questions remain, however about whether and how Notch signaling regulates immune cells other than T and B cells. To the best of our knowledge, there are only two reports describing the relationship between Notch signaling and mast cells. According to these papers, Jagged-1, a Notch ligand (8), and Notch2 (9), are highly expressed in mast cells.

Here we demonstrate that Notch signaling has a significant role in mast cell development. Using *Notch2*–null bone marrow cells, we show that Notch2 signaling promotes cell fate determination of bone marrow progenitors toward the progenitors having mast cell differentiation capacity at the expense of those that lose it. Furthermore, we demonstrate that the coordinated regulation of two transcription factors, Hes-1 and GATA3, determines mast cell fate under Notch signaling. Overexpression of Hes-1 in myeloid progenitors represses C/EBPα mRNA expression, in agreement with the

proposal that the C/EBPα downregulation provides essential condition for mast cell development (5).

These findings suggest that Notch signaling, a key regulator of lymphocytes, more broadly affects cells of the immune system through dynamic regulation of transcription factors.

Results Notch2-mediated signaling facilitates mast cell lineage development at the expense of granulocyte/macrophage development from both CMPs and GMPs *in vitro*

We cultured isolated CMPs and GMPs in SCF, IL-3, IL-6, and TPO for 7 d with plate-fixed Delta1-Fc chimeric protein, a soluble Notch ligand. In the culture using control Fc protein, instead of Delta1-Fc, the vast majority of the cells were $Gr1^{+}Mac1^{+}$ granulocytes/macrophages Compared with this, $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{+}$ mast cells were highly enriched, while there were fewer $Gr1^{+}$ or $Mac1^{+}$ granulocytes/macrophages with Delta1-Fc (Fig. 1A). The cell number was not significantly different in the culture with Delta1-Fc from the culture with the control Fc protein on day 7, when the flowcytometric analysis was performed (Fig. 1B). As shown, time courses of cell growth were different between the cultures with Delta1-Fc and control, due to the difference in the differentiation fate. Morphological observation confirmed the conversion from granulocytes/macrophages with the control Fc to mast cells, which contained toluidine blue-positive granules, with Delta1-Fc (Fig. 1C). The expression level of mast cell-specific protease, mast cell protease-5 (mMCP5), was higher in Delta1-stimulted population compared with control (Fig. 1D).

While all of Notch1, Notch2 and Notch3 are expressed in myeloid progenitors

(Supplementary Fig. 1), Notch2 is known to be expressed in mast cells amongst Notch 1-4 receptors (9). We sorted CMPs and GMPs from polyinosinic-polycytidylic ribonucleic acid (pIpC)-treated Mx-Cre x *Notch2*$^{flox/flox}$ (N2-MxcKO) mice (10), and then stimulated with Delta1-Fc. The mast cell increase by Delta1-Fc was strikingly blunted with *Notch2*-null CMPs or GMPs, whereas the results obtained with littermate *Notch2*$^{flox/flox}$ mice treated with pIpC were virtually the same as those obtained with wild-type mice (Fig. 1E; compare with Fig. 1A). These observations indicate that Notch2-mediated signaling plays a key role in derivation of mast cells from myeloid progenitor cells.

Hes-1 is upregulated downstream of Notch signaling but insufficient for mast cell derivation

Quantitative real-time PCR analysis revealed that Delta1-Fc treatment of CMPs or GMPs upregulated mRNA for Hes-1, a basic helix-loop-helix transcriptional repressor that often functions downstream of Notch signaling (11), within as little as 8 h (Fig. 2A). To investigate whether Hes-1 is a downstream effector of Notch signaling for mast cell enrichment by Delta1-Fc, we retrovirally expressed Hes-1 in CMPs and GMPs. Expression of the Hes-1 protein was confirmed by a western blot analysis of the

retrovirally infected NIH-3T3 cells (Fig. 2B). Derivation of the $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{+}$ mast cells, however, was not increased by Hes-1 expression after 8 d of culture (Fig. 2C), whereas there was a relative increase in $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{-}$ cells. This observation indicated that the effect of Hes-1 expression on mast cell enrichment was not equivalent to that of Delta1-Fc stimulation, suggesting the presence of an effector molecule other than Hes-1 at the downstream of Notch.

GATA3, but not GATA2, is a mediator downstream of Notch2 for mast cell developmental decision, together with Hes-1

Among the GATA family of transcription factors, GATA2, which is upregulated during Notch-mediated inhibition of granulocytic differentiation (12, 13), is proposed to be a regulator of mast cell differentiation (4, 5, 14). Nevertheless, GATA2 expression levels in CMPs or GMPs stimulated with Delta1-Fc were equal to or lower than those stimulated with control Fc protein during the first 48 h (Fig. 3A).

GATA3 is a crucial transcription factor during T cell development. Recently, it was reported that its overexpression in double negative (DN) 1 and DN2 stage thymocytes blocked further T cell development, and unexpectedly, induced mast cells from these early thymocyte populations (15). Physiologic relevance between GATA3

and mast cell generation, however, is yet to be elucidated. Delta1-Fc stimulation of CMPs and GMPs substantially increased GATA3 mRNA during a relatively later time course (Fig. 3B). This finding suggests that GATA3 is a possible target of Notch signaling, albeit indirect, for mast cell production.

Thus, we examined whether Hes-1 and GATA3 are bona fide effectors functioning downstream of Notch signaling for mast cell production, by retrovirally transducing CMPs and GMPs with these genes (Fig, 3C and D). Both CMP- and GMP-derived fractions expressing Hes-1 alone (with the NGFR marker) gave rise to populations very similar to those derived from the fraction expressing Hes-1 with the GFP marker (Fig. 2C, 3C, and 3D). There was no consistent increase in the c-Kit$^+$FcεRI$^+$ mast cell population at day 8, although it appears that Hes-1 single positive CMP-derived fraction gave rise to this population slightly more than control in Fig. 3C and D. On the contrary, the Hes-1 single positive fractions gave rise to fewer mast cells than the control virus-infected fractions at the later time course (data not shown). In the fractions expressing GATA3 alone (with the GFP marker), the c-Kit$^+$FcεRI$^+$ mast cell population only marginally, although reproducibly, increased compared with controls (Fig. 3C and D).

In contrast to these, the c-Kit$^+$FcεRI$^+$ mast cell population was markedly

enriched in the Hes-1 and GATA3 double positive fractions at day 8 after the infection of CMPs and GMPs compared with either mock, or Hes-1 or GATA3 single positive fractions (Fig. 3C and D).

We reproducibly observed increase in the c-Kit^{+}FcεRI^{-} population, which might represent the expanded progenitors maintaining the immature state, in the Hes-1 single positive fractions. The c-Kit^{-}FcεRI^{+} population, whose identification is unknown, variably increased in the GATA3 single positive fractions compared with controls (Fig. 3C).

When plated in methylcellulose after the retroviral infection, Hes-1- and GATA3-co-expressing CMPs and GMPs formed large and monotonous cell-containing colonies (plating efficiency, ~20% in CMPs and <10% in GMPs); staining indicated that more than 80% of colonies contained mast cells, positive for toluidine blue, as the major population (Fig. 3 E, F, and G), while the plating efficiency was comparable to that with mock introduction (Fig. 3E).

These observations indicate that the simultaneous expression of GATA3 and Hes-1 biased the cell fate of myeloid progenitors toward the downstream progenitors having mast cell-generating capacity at the single cell level, rather than that those transcription factors expanded the pool of mast cell progenitors or mast cells.

Hes-1 upregulation causes C/EBPα downregulation C/EBPα is a critical transcription factor for myeloid differentiation (16), and its downregulation cooperates with the upregulation of a GATA transcription factor to instruct mast cell development (5). At 48 h after the initiation of Hes-1 retroviral transduction, C/EBPα mRNA was downregulated in both CMPs and GMPs (Fig. 4A). The C/EBPα mRNA level was also markedly suppressed in a mouse myeloid cell line 32D that stably expressed exogenous Hes-1 (Fig. 4B). These findings suggest that the C/EBPα downregulation is the major outcome of Hes-1 upregulation induced by Delta1-Notch2 signaling in the myeloid progenitors.

Discussion

In the present study, we demonstrate that Notch2-mediated signaling has a significant role in the mast cell derivation from myeloid progenitors such as CMPs and GMPs. This biological effect is probably mediated through coordinated upregulation of Hes-1 and GATA3 through Notch2 signaling; Hes-1 upregulation further results in C/EBPα downregulation, which is important for the blockade of myeloid lineage differentiation. It is not unexpected that Hes-1 upregulation plays a role in the execution of Notch2

signaling, given the fact that Hes-1 is an established target of Notch signaling in a number of cell systems (17). However, we show in this paper that Hes-1 upregulation represents only a part of signaling downstream of Notch activation. Exogenous Hes-1 expression in CMPs and GMPs resulted in the increase of $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{-}$ cells; obviously different from the $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{+}$ mast cell enrichment that was seen by the Delta1 stimulation. Although the identity of the $Lin^{-}c\text{-}Kit^{+}Fc\varepsilon RI^{-}$ cells is yet to be determined, these cells may contain immature myeloid progenitors which could differentiate into mast cells if GATA3 would co-exist, while granulocytes/macrophages if other critical molecules (such as C/EBPα) would.

We identified GATA3, not GATA2, upregulation that complements Hes-1 upregulation and creates a part of Notch signaling. Involvement of GATA2 is proposed to be important for inhibiting granulocytic differentiation downstream of Notch signaling in both the 32D cell line (13) and mouse hematopoietic progenitor cells (12). Furthermore, GATA2 is required for *in vitro* mast cell generation (4, 14), and enforced expression of GATA2 instructs the C/EBPα-deficient myeloid progenitors as well as common lymphoid progenitors to become mast cells *in vitro* (5). Our conclusion might appear to be inconsistent with these lines of papers. We confirmed that the enforced GATA2 and Hes-1 co-expression in CMPs and GMPs resulted in predominant mast cell

generation in a manner indistinguishable from that of GATA3 and Hes-1 co-expression (data not shown). This indicates that GATA2 and GATA3 have redundant properties when they are expressed exogenously. The result of colony formation assay from CMPs and GMPs with enforced GATA3 and Hes-1 expression indicates that the mast cell derivation is based on the cell fate alteration made in the individual progenitor cells. Because the biological readout for Notch ligand stimulation was virtually the same as that for the GATA3 and Hes-1 co-expression, the substantial mast cell generation at day 7 with Delta1-Fc is likely to be mainly caused by biased cell fate decision in the myeloid progenitors.

In a different line, the introduction of GATA3 alone to thymocytes was recently reported to result in mast cell generation (15). Although this report might appear to be inconsistent with our data, the difference in the starting cell populations could cause the different results. We conclude that the Hes-1 expression, and probably subsequent C/EBPα downregulation, is required, though not sufficient, for mast cell generation from CMPs and GMPs. In contrast, C/EBPα is already downregulated in thymocytes at the DN1 and DN2 stages (18), and thus, introduction of GATA3 might be sufficient for mast cell generation from early thymocytes. As for the relationship between GATA3 and Notch signaling, both Notch1 and Notch2 are important for the generation of Th2

cells, and act by directly inducing transcription of GATA3 and IL-4 (19, 20) It is, however, unclear whether such a direct regulation is applicable to cells in other lineages. In our observation, GATA3 was upregulated by Delta1-Fc at 48 h but not at 8 h in CMPs and GMPs, making it obscure whether GATA3 is a direct target of Notch signaling in these cells, while the role of IL-4 in the regulation of GATA3 remains to be determined.

We and others previously reported that enforced expression of Hes-1 in a 32D cell line inhibits granulocytic differentiation induced by granulocyte colony-stimulating factor (13, 21). In the present study, we demonstrated that C/EBPα downregulation occurs downstream of Hes-1 in both the 32D cell line and fresh CMPs and GMPs. C/EBPα repression by Hes-1 was previously shown to be among the mechanisms of Notch-Hes-1-mediated inhibition of adipogenesis from a preadipocyte cell line (22). Although less remarkable compared with Hes-1 overexpression, Delta1-Fc stimulation also induced C/EBPα repression, in a time course after Hes-1 upregulation. This suggests that the physiologic Hes-1 up-regulation is sufficient for C/EBPα repression (data not shown). Our data, thus, support a new paradigm that the Notch-Hes-1-C/EBPα axis consists of a common pathway for differentiation inhibition in a variety of cell lineages.

It was recently suggested that downregulation of C/EBPα followed by upregulation of a GATA factor orchestrates mast cell differentiation from myeloid progenitors (5). This could be true, but importantly, we demonstrated that such a balanced regulation of transcription factors is a result of environmental signaling through Notch2, rather than a cell-autonomous operation (Fig. 4C).

The physiological significance of Notch2-mediated cell fate bias toward mast cell lineage remains to be determined, because mast cells were not depleted in naïve status in N2-MxcKO mice (M. S.-Y. and S.C., unpublished data). Cultured mast cells were also generated from Notch2-null bone marrow cells as efficiently as wild-type bone marrow cells. Notably, mast cells are not depleted in mice lacking IL-3 gene, while IL-3 is the most potent mast cell developmental factor *in vitro*. However, IL-3-deficient mice are defective in mast cell-mediated intestinal nematode eradication. The pathways and mechanisms responsible for regulating mast cell progenitor recruitment and trafficking are likely to be dynamic and susceptible to modification during inflammation (1). Similarly, Notch2 is required for the proper resonse of mast cells during nematode infection. (M.S.-Y. and S.C.; unpublished observation.) Notch2-mediated mast cell derivation might also be required for such pathological settings, while it is unnecessary for the steady state mast cell generation.

Materials and Methods

Mice

Notch2$^{flox/flox}$ mice were described previously (10). Mx-Cre transgenic mice (23) were crossed with *Notch2*$^{flox/flox}$ mice and the progeny were injected with pIpC (Sigma-Aldrich) 7 times every other day from 3 d after birth (25 μg/g body weight) or 3 times between 4 and 6 wk of age (20 μg/g body weight). All experiments were done in accordance with our Institutional Guidelines.

Myeloid progenitors

Bone marrow cells from each mouse strain studied were incubated with biotinylated antibodies for lineage markers including anti-CD3, anti-CD4, anti-CD8, anti-B220, anti-Ter119, and anti-Gr-1 antibodies (BD Pharmingen) followed by incubation with streptavidin Micro Beads (Miltenyi Biotec). The lineage marker-negative fraction was separated with an autoMACS separator (Miltenyi Biotec), and incubated with anti-CD34-FITC, anti-CD16/32 (FcγIII/II receptor)-PE, anti-c-Kit-APC, streptavidin PerCP (BD Pharmingen), and anti-Sca1-PE/Cy7 (eBioscience).

$Lin^-c\text{-}Kit^+Sca1^-Fc\gamma R^{lo}CD34^+$ and $Lin\text{-}c\text{-}Kit^+Sca1^-Fc\gamma R^{hi}CD34^+$ cells (CMPs and GMPs, respectively (24) were sorted by a FACSAria cell sorter (Becton Dickinson).

Ligand fixation

Delta1-Fc was described previously (25). A 24-well non-tissue culture plate (Nalge Nunc) was coated with 10 μg/mL of rabbit anti-human IgG (DAKO), blocked with 20% fetal bovine serum (FBS) -containing RPMI-1640 medium (Sigma-Aldrich), and washed with phosphate-buffered saline. The Delta1-Fc (3.5 μg/mL) or Fc portion of human IgG (2 μg/mL, Fc protein, ART or Jackson Immuno Research Laboratories) was then incubated for 30 min and supernatants were removed.

Ligand stimulation of myeloid progenitors

Sorted CMPs or GMPs were cultured in Delta1-Fc or control Fc protein-fixed plates in 20% FBS containing Iscove's modified Dulbecco's medium (Sigma-Aldrich), supplemented with 50 ng/mL SCF, 20 ng/mL IL-3 (Peprotech), 20 ng/mL IL-6, and 20 ng/mL TPO (gifts from Kirin Brewery Research Laboratory). On day 7, the cells were incubated with purified isotype IgE (BD Pharmingen) after blocking the Fcγ receptor with anti-CD16/32 (Fcγ III/II receptor) antibody (BD Pharmingen), stained with

anit-IgE-FITC, -Gr-1-PE, -Mac1-PE (BD Pharmingen), and -c-Kit-APC, and then analyzed by FACSCalibur (Becton Dickinson). Cells cultured for 7 days were also characterized by Wright-Giemsa staining or toluidine blue staining (pH 0.5) on cytospin slides. In some experiments, mRNA was prepared at the indicated time points and quantified by real-time PCR, as described below.

Retroviral transduction

Hes-1 cDNA, a gift from R. Kageyama, was subcloned into a retrovirus vector, GCDNsam/IRES-nerve growth factor receptor (NGFR), a gift from H. Nakauchi and M. Onodera. cDNAs for GATA3, a gift from S. Takahashi, and Hes-1 were subcloned into pMYs/IRES-GFP, a gift from T. Kitamura. A retrovirus packaging cell line, PLAT-E (26), was transfected with each retrovirus vector using FuGENE 6 (Roche Diagnostics). The conditioned medium was concentrated and placed in a 24-well non-tissue culture dish for 4 h, pre-coated with 40 μg/mL of RetroNectin (Takara Bio) overnight at 4°C. Sorted CMPs or GMPs were then plated for infection in the presence of 20% FBS, 50 ng/mL SCF, 20 ng/mL IL-3, 20 ng/mL IL-6, and 20 ng/mL TPO. Green fluorescent proteins- and/or human NGFR-positive fractions were subjected to FACS analysis 8 d after infection. Otherwise, the infected cells were sorted at 48 h from the initiation of

infection with a FACSAria (Becton-Dickinson) cell sorter and used for colony assay using Methocult M3231 (StemCell Technologies), supplemented with cytokines as described above Hes-1 stably expressing 32D cell lines were previously described (13). To confirm the protein expression, NIH-3T3 cells were also infected with the same viral supernatants using polybrene (Sigma-Aldrich).

RNA quantitation

Total cellular RNA was extracted with RNeasy (Qiagen) and converted to cDNA with Superscript III (Invitrogen). GATA2, GATA3, C/EBPα, and mouse mast cell progease-5 (mMCP-5) were analyzed with TaqMan Gene Expression assays (Applied Biosystems). Hes-1 mRNA was measured as previously described (10). Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All the data were standardized with the 18s ribosomal RNA.

Western blot analysis

Virus-infected NIH-3T3 cells were solubilized in lysis buffer containing 1% Triton X-100. The Hes-1 and GATA3 proteins were detected by anti-Hes-1 antibody (H-140, Santa Cruz) and anti-GATA3 antibody (HG3-31), respectively.

Statistical analysis

Results from two or three independent experiments (n=2) of quantitative real-time PCR were analyzed by the Mann-Whitney test.

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Authorship

MSY designed and performed research, analyzed data, and wrote the paper. ENY performed research. TS, KK, KY, SO, and MK contributed to the work through vital discussion. SC was involved in designing research and wrote the paper with MSY. Correspondence: Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan; e-mail: schiba-tky@umin.net.

Figure Legends

Fig.1 Notch2-mediated signaling facilitates mast cell lineage development at the expense of granulocyte/macrophage development from CMPs and GMPs *in vitro*. A, Sorted CMPs or GMPs of wild-type mice were cultured with plate-fixed Delta1-Fc chimeric protein or control Fc protein for 7 d in 50 ng/mL SCF, 20 ng/mL IL-3, 20 ng/mL IL-6, and 20 ng/mL TPO. FACS analysis showed $Fc\varepsilon RI^{+}$ c-Kit^{+} $Gr1^{-}$ $Mac1^{-}$ mast cells were highly enriched with Delta1-Fc, while $Gr1^{+}$ $Mac1^{+}$ granolocytes and macophages were main population with control Fc protein.
B, Cell growth in culture of CMPs and GMPs with Delta1-Fc or control Fc protein.

C, Day 7 progeny of CMPs and GMPs were analyzed morphologically by cytospin. (Left panel) Wright-Giemsa staining; original magnification, x400. (Right panel) Toluidine blue staining; original magnification, x400. **D,** mRNA was extracted from day 7 progeny of CMPs and GMPs, reverse-transcribed and applied to quantitative real-time PCR analysis for mouse mast cell protease-5 (mMCP-5). Data are presented as mean +/- SD; n=4, p=0.0286 [CMPs, 7d]; n=4, p=0.0286 [GMPs, 7d] (non parametric test). **E,** CMPs and GMPs of Mx-Cre x *Notch2*$^{flox/flox}$ and littermate *Notch2*$^{flox/flox}$ mice were cultured with Delta1-Fc or control Fc protein for 7 d in SCF, IL-3, IL-6, and TPO.

Notch2-null CMPs and GMPs failed to respond to Delta1-Fc, whereas the results obtained with littermate mice treated with pIpC were virtually the same as those obtained with wild-type mice.

Fig.2 Hes-1 upregulation downstream of Notch signaling is insufficient for mast cell derivation A, CMPs and GMPs were stimulated with plate-fixed Delta1-Fc protein or control Fc protein in SCF, IL-3, IL-6, and TPO. Cells were collected at 8 h and Hes-1 mRNA was measured by quantitative real-time PCR analysis. Hes1 mRNA was substantially upregulated with Delta1-Fc compared with control. Data are presented as mean +/- SD; n=4, p=0.0286 [CMPs, 8h]; n=4, p=0.0286 [GMPs, 8h] (non parametric test). **B,** A retrovirus packaging cell line, PLAT-E, was transfected with cDNA-subcloned retrovirus vectors, i.e., Hes-1-GCDNsam/IRES-nerve growth factor receptor (NGFR), Hes-1-pMYs/IRES-GFP, and GATA3-pMYs/IRES-GFP, or a mock retrovirus vector. NIH-3T3 cells were infected with the conditioned medium using polybrene. The proteins were detected by anti-Hes-1 antibody (H-140, Santa Cruz) and anti-GATA3 antibody (HG3-31). **C,** CMPs and GMPs were retrovirally transduced with Hes-1-pMYs/IRES-GFP or mock

virus. The proportion of c-Kit^{+}FcεRI^{+} mast cells was not significantly increased by Hes-1 expression. The proportion of c-Kit^{+}FcεRI^{-} cells, which probably represent myeloid progenitors, was always greater in the Hes-1-expressing population than control.

Fig.3 GATA3, but not GATA2, is a mediator downstream of Notch2 for mast cell developmental decision, together with Hes-1 A, CMPs and GMPs were stimulated with plate-fixed Delta1-Fc in the presence of stem cell factor, interleukin (IL) -3, IL-6, and thrombopoietin for 8 h or 48 h. Cells were harvested and quantitative real-time PCR was performed. GATA2 mRNA levels were slightly decreased rather than increased by Delta1-Fc in GMPs at 8 h, and not significantly different in CMPs treated with Delta1-Fc and control Fc at 8 h or in CMPs and GMPs treated with Delta1-Fc and control Fc at 48 h. **B,** Quantitative real-time PCR analyses in CMPs and GMPs revealed that GATA3 mRNA was upregulated by Delta1-Fc at 48 h, but not at 8 h. Data are presented as mean +/- SD; n=4, p=0.6857 [CMPs, 8 h]; n=4, p=0.0571 [GMPs, 8 h]; n=4, p=0.0286 [both CMPs and GMPs, 48 h] (non parametric test). **C**, CMPs and GMPs were retrovirally transduced with Hes-1-GCDNsam/IRES-NGFR

and GATA3-pMYs/IRES-GFP, and subjected to FACS analysis 8 d after the infection.

The c-Kit^{+}FcεRI^{+} fraction was remarkably enriched in Hes-1 and GATA3 double positive fraction compared with the Hes-1 or GATA3 single positive fraction, or the mock virus-introduced fraction. **D,** The proportions of c-Kit^{+}FcεRI^{+} fraction are depicted. Each diamond represents a data point. The bars represent +/- 2SD. CMP n=6; GMP n=4. **E,** NGFR and GFP double positive cells were sorted at 48 h after infection. Two hundred and fifty NGFR and GFP double positive CMPs and 1500 GMPs were plated per dish and cultured for 7 d in methylcellulose, supplemented with SCF, IL-3, IL-6, and TPO. Hes-1- and GATA3-co-expressing cells formed mainly mast cell colonies, while mock virus transduced cells formed various colonies including granulocyte, macrophage or mixture of these cells. G, granulocyte colonies; M, macrophage colonies; mix, granulocyte/macrophage or mixed colonies; *, colonies mainly consisting of mast cells. The result of a representative experiment is shown (n=2). **F,** Wright-Giemsa staining of Hes-1 and GATA3 co-expressing colony-forming cells**.** Original magnification, x400. **G,** Toluidine blue staining of Hes-1 and GATA3 co-expressing colony-forming cells. Original magnification, x400.

Fig.4 Hes-1 upregulation causes C/EBPα downregulation A, CMPs and GMPs were retrovirally transduced with Hes-1-pMYs/IRES-GFP. GFP^+ cells were sorted 48 h after infection and were examined for C/EBPα mRNA by quantitative real-time PCR. C/EBPα mRNA in CMPs and GMPs was substantially reduced by Hes1 compared with mock virus transduction. Data are presented as mean +/- SD; n=5 [mock] or 4 [Hes-1], p=0.0159 [CMP]; n=5, p=0.0079 [GMP] (non parametric test). **B,** 32D cells stably transduced with Hes-1 and maintained in 5 ng/mL IL-3 were examined for C/EBPα expression. C/EBPα mRNA was reduced in Hes1-transduced clones compared with mock-transduced clones. Data were confirmed by experiments using two independent clones. Data are presented as mean +/- SD. **C,** Schematic model of Notch signaling in the mast cell system.

References

1. Gurish M-F, Boyce J-A (2006) Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. J Allergy Clin Immunol 117: 1285-91.

2. Galli S-J, Nakae S, Tsai M (2005) Mast cells in the development of adaptive immune responses. Nat Immunol 6: 135-42.

3. Shelburne C-P, et al (2002) Stat5: an essential regulator of mast cell biology. Mol Immunol 38: 1187-91.

4. Walsh J-C, et al (2002) Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. Immunity 17: 665-76.

5. Iwasaki H, et al (2006) The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. Genes Dev 20: 3010-21.

6. Radtke F, Wilson A, Mancini S-J, MacDonald H-R (2004) Notch regulation of lymphocyte development and function. Nat Immunol 5: 247-53.

7. Tsukumo S, Yasutomo K (2004) Notch governing mature T cell differentiation. J Immunol 173: 7109-13.

8. Singh N, Phillips R-A, Iscove N-N, Egan S-E (2000) Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. Exp Hematol 28: 527-34.

9. Jonsson J-I, Xiang Z, Pettersson M, Lardelli M, Nilsson G (2001) Distinct and regulated expression of Notch receptors in hematopoietic lineages and during myeloid differentiation. Eur J Immunol 31: 3240-7.

10. Saito T, et al (2003) Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity 18: 675-85.

11. Jarriault S, et al (1995) Signalling downstream of activated mammalian Notch. Nature 377: 355-8.

12. de Pooter R-F, et al (2006) Notch signaling requires GATA-2 to inhibit myelopoiesis from embryonic stem cells and primary hemopoietic progenitors. J Immunol 176: 5267-75.

13. Kumano K, et al (2001) Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. Blood 98: 3283-9.

14. Tsai F-Y, Orkin S-H (1997) Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 89: 3636-43.

15. Taghon T, Yui M-A, Rothenberg E-V (2007) Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. Nat Immunol 8: 845-855.

16. Zhang P, et al (2004) Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity 21: 853-63.

17. Kageyama R, Ohtsuka T, Tomita K (2000) The bHLH gene Hes1 regulates differentiation of multiple cell types. Mol Cells 10: 1-7.

18. Laiosa C-V, Stadtfeld M, Xie H, de Andres-Aguayo L, Graf T (2006) Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. Immunity 25: 731-44.

19. Amsen D, et al (2007) Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. Immunity 27: 89-99.

20. Fang T C, et al (2007) Notch directly regulates Gata3 expression during T helper 2 cell differentiation. Immunity 27: 100-10.

21. Tan-Pertel H-T, et al (2000) Notch signaling enhances survival and alters differentiation of 32D myeloblasts. J Immunol 165: 4428-36.

22. Ross D-A, Rao P-K, Kadesch T (2004) Dual roles for the Notch target gene Hes-1 in the differentiation of 3T3-L1 preadipocytes. Mol Cell Biol 24: 3505-13.

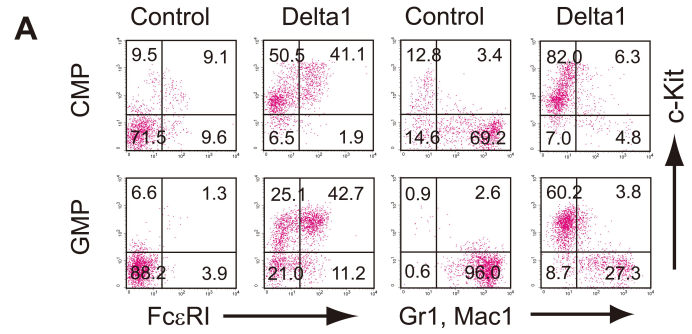
23. Kuhn R, Schwenk F, Aguet M, Rajewsky K (1995) Inducible gene targeting in mice. Science 269: 1427-9.

24. Akashi K, Traver D, Miyamoto T, Weissman I-L (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404: 193-7.

25. Shimizu K, et al (1999) Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. J Biol Chem 274: 32961-9.

26. Kitamura T, et al (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol 31: 1007-14.

Fig.1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12.8
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82.0
6.3
7.0
4.8
6.6
1.3
88.2
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42.7
21.0
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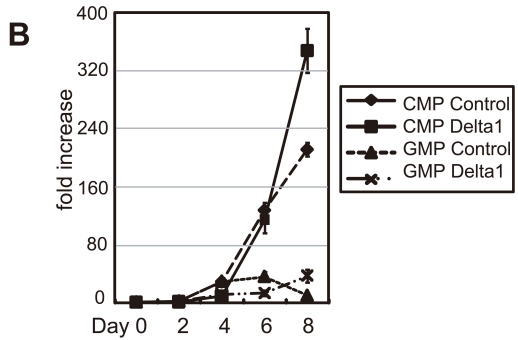
c-Kit
FcεRI
Gr1, Mac1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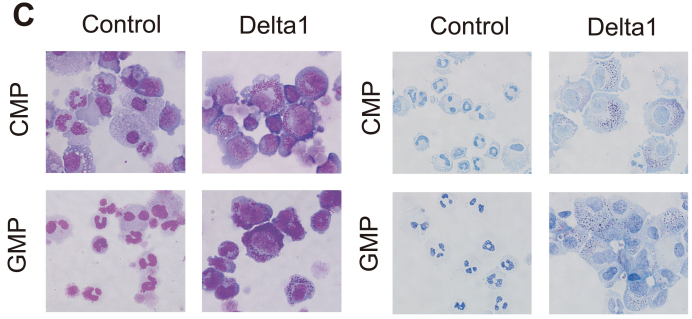
0
fold increase
Day 0
2
4
6
8
CMP Control
CMP Delta1
GMP Control
GMP Delta1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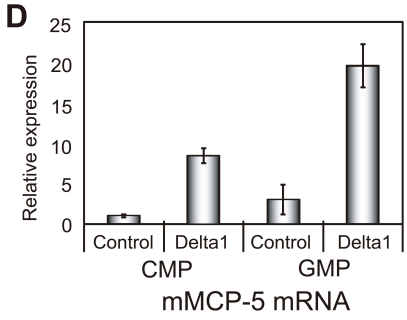
CMP
GMP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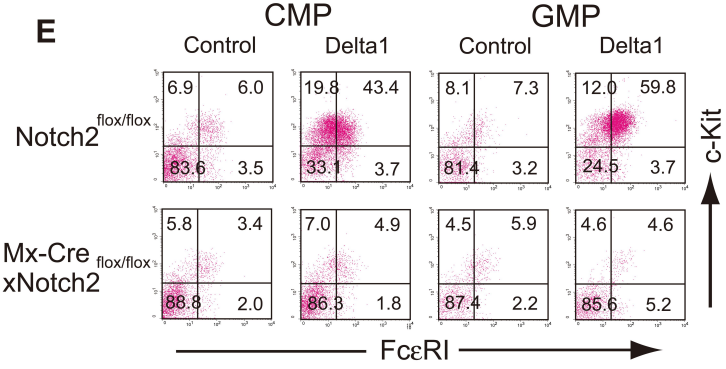
25
20
15
10
5
0
Relative expression
Control
Delta1
Control
Delta1
CMP
GMP
mMCP-5 mRNA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GMP
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19.8
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33.1
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7.3
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3.2
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7.0
4.9
86.3
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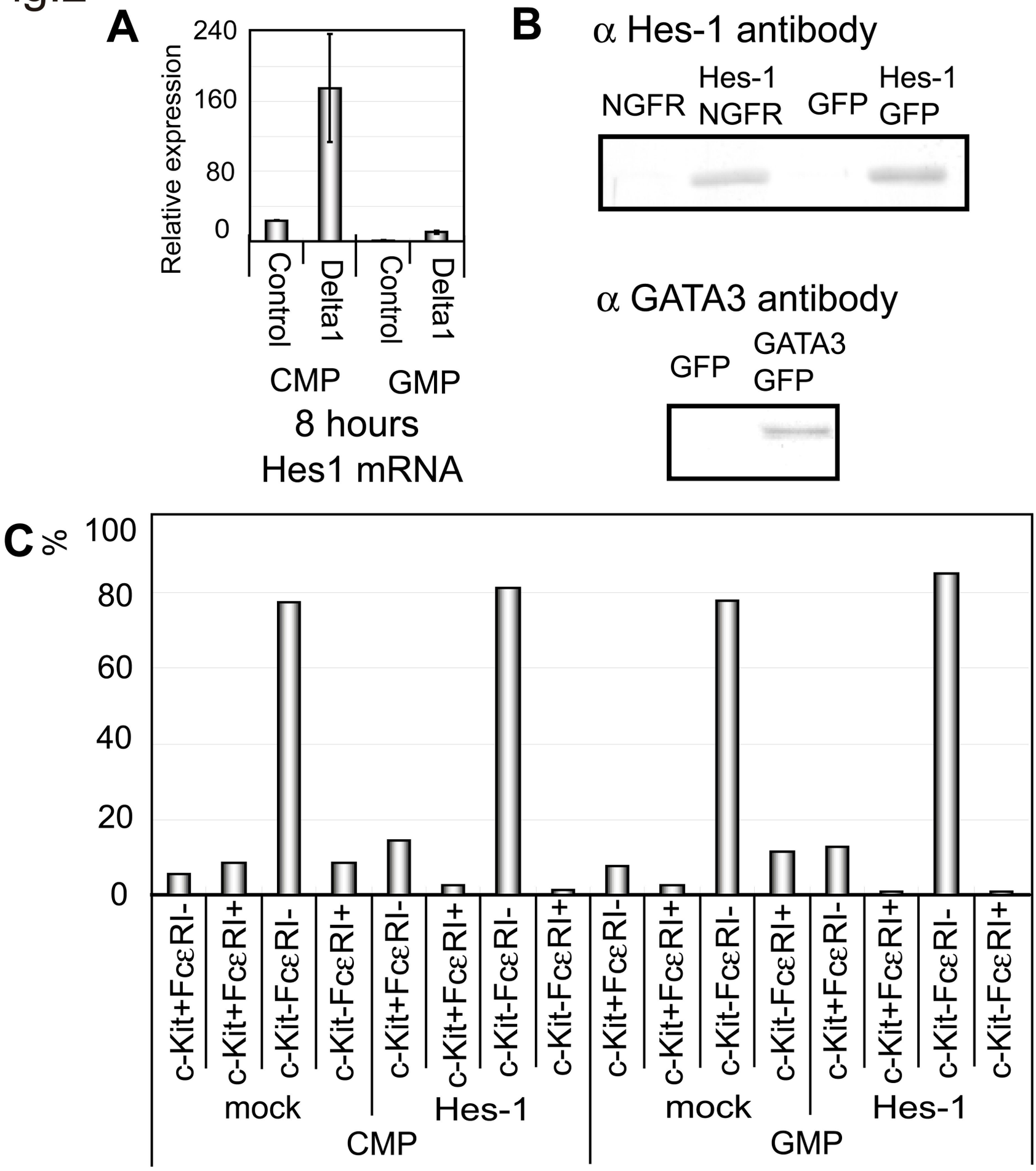
5.2
c-Kit
FcεRI

Fig.2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240
160
80
0
Relative expression
Control
Delta1
Control
Delta1
CMP
GMP
8 hours
Hes1 mRNA
B
α Hes-1 antibody
NGFR
Hes-1 NGFR
GFP
Hes-1 GFP
α GATA3 antibody
GFP
GATA3 GFP
C
%
100
80
60
40
20
0
c-Kit+FcεRI-
c-Kit+FcεRI+
c-Kit-FcεRI-
c-Kit-FcεRI+
mock
Hes-1
CMP
GMP

Fig.3

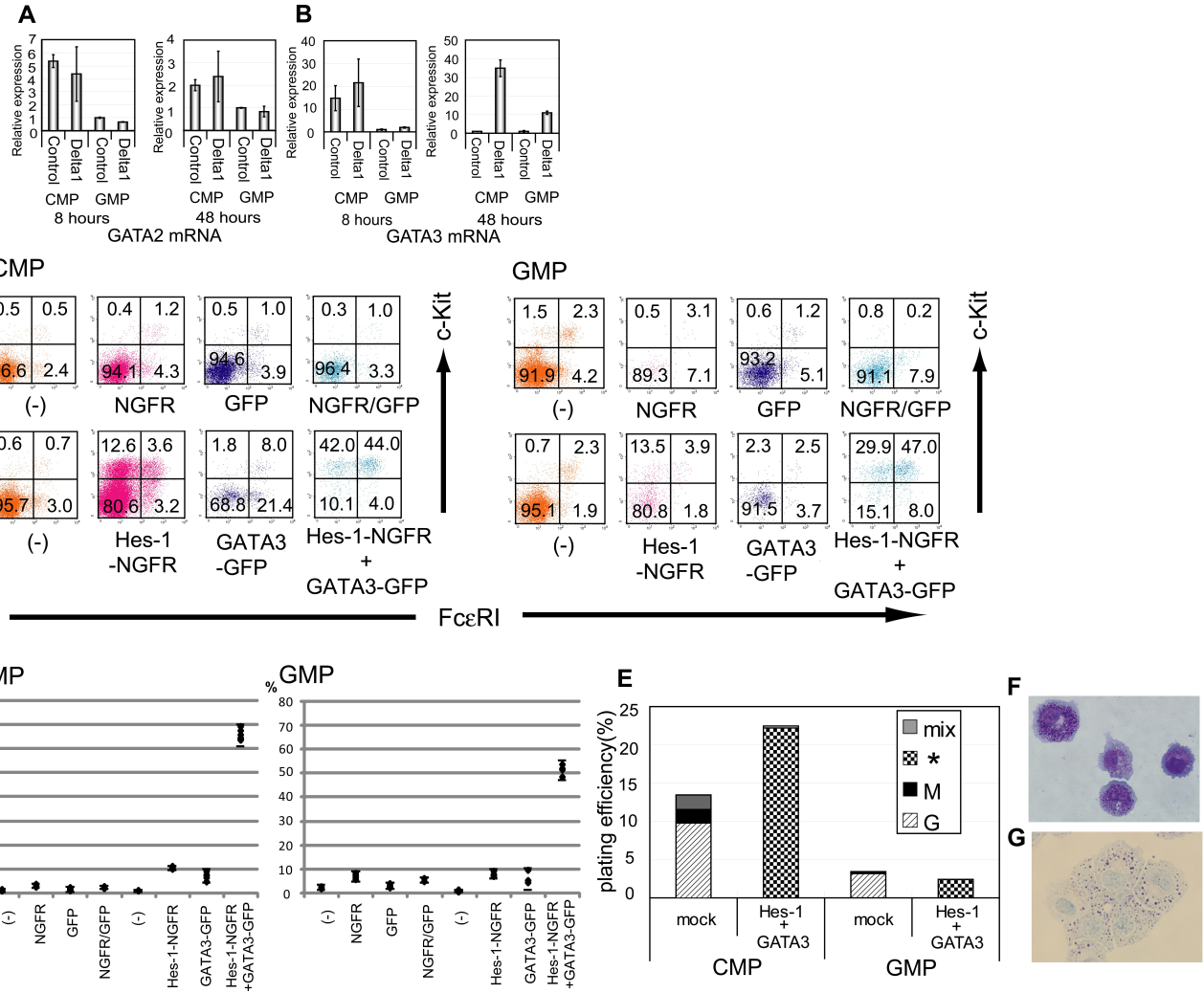
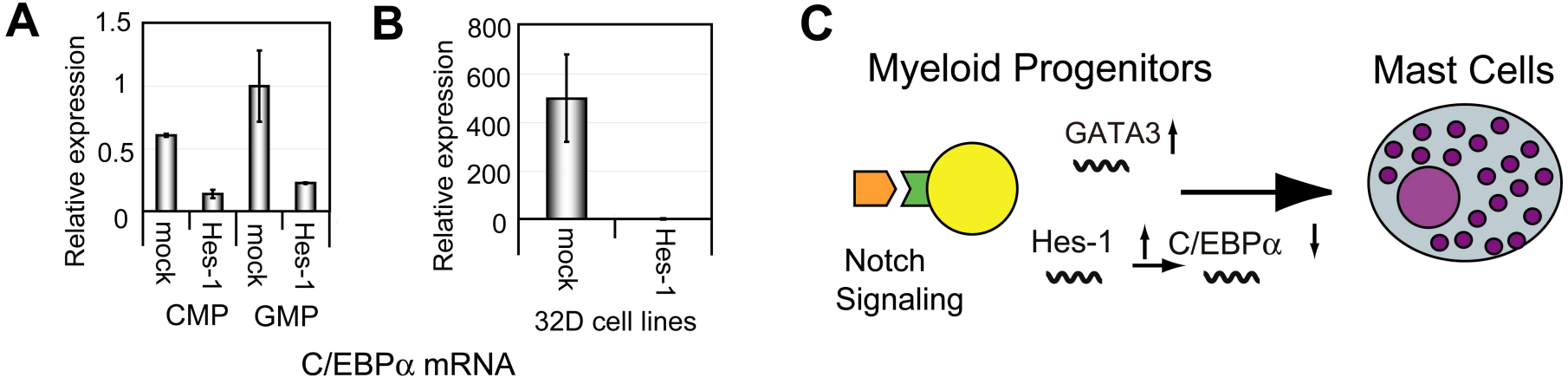

A
B
Relative expression
Control
Delta1
CMP
GMP
8 hours
48 hours
GATA2 mRNA
GATA3 mRNA
C
CMP
GMP
c-Kit
(-)
NGFR
GFP
NGFR/GFP
Hes-1 -NGFR
GATA3 -GFP
Hes-1-NGFR + GATA3-GFP
FcεRI
D
%
(-)
NGFR
GFP
NGFR/GFP
Hes-1-NGFR
GATA3-GFP
Hes-1-NGFR +GATA3-GFP
E
plating efficiency(%)
mix
*
M
G
mock
Hes-1 + GATA3
F
G

Fig.4


A
Relative expression
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1
0.5
0
mock
Hes-1
mock
Hes-1
CMP
GMP
B
Relative expression
800
600
400
200
0
mock
Hes-1
32D cell lines
C/EBPα mRNA
C
Myeloid Progenitors
Mast Cells
GATA3
Notch
Signaling
Hes-1
C/EBPα